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With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: IL-2 DELETION MUTANTS

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                                1                                  10
          IL-2 amino acid:  Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu Gln
            cDNA sequence:  gca cct act tca agt tct aca aag aaa aca cag cta caa
       codon modifications:                 t   c       c           c       g   g
synthetic DNA sequence:  GCA CCT ACT TCT AGC TCT ACC AAG AAA ACC CAG CTG CAG

                           20                                  30
Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile Asn Asn Tyr Lys Asn
ctg gag cat tta ctg ctg gat tta cag alg att ttg aat gga att aat aat tac aag aat
      c c g               g           c c   c   t   c   c               c
CTC GAG CAC CTG CTG CTG GAT TTG CAG ATG ATC CTG AAC GGT ATC AAC AAT TAC AAG AAC
 XhoI

                           40                                  50
Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala Thr Glu Leu
ccc aaa ctc acc agg atg ctc aca ttt aag ttt tac atg ccc aag aag gcc aca gaa ctg
  g       g     c t       g   c   c       c       g               c
CCG AAA CTG ACG CGT ATG CTG ACC TTC AAG TTC TAC ATG CCG AAG AAG GCC ACC GAA CTG
          MluI

                           60                                  70
Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn Leu Ala
aaa cat ctt cag tgt cta gaa gaa gaa ctc aaa cct ctg gag gaa gtg cta aat tta gct
    c   g           g           g       g               t   g   c c g
AAA CAC CTG GAG TGT CTA GAA GAA GAA CTG AAA CCG CTG GAG GAA GTT CTG AAC CTG GCT
                  XbaI
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(57) Abstract

A mutant IL-2 molecule capable of binding an IL-2 receptor-bearing cell, having a deletion of one to five amino acid residues of IL-2, the deletion resulting in active IL-2 molecules that have increased resistance to proteolysis.

* See back of page

DESIGNATIONS OF "DE"

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WO 91/02000 PCT/US90/04258

IL-2 DELETION MUTANTS

Background of the Invention

This invention relates to the use of recombinant DNA techniques to make mutant interleukin-2 (IL-2, molecules and chimeric IL-2/toxin molecules.

Il-2 is a protein secreted by human T-lymphocytes which is capable of binding to IL-2 receptors on activated T-lymphocytes and effecting T-lymphocyte proliferation. IL-2 has been shown to be a therapeutic immunostimulant in humans (Rosenberg, 1988, Immunology Today 9:2: 58-62), and IL-2 or a specific binding portion thereof can be coupled to the enzymatically active portion of diphtheria toxin to form a hybrid molecule with a number of therapeutic applications (Murphy U.S. Patent No. 4,675,382, hereby incorporated by reference). IL-2/diphtheria toxin hybrid proteins of Murphy '382, which were made using recombinant DNA techniques, have been shown to inhibit rejection of transplanted organs (Pankewycz et al., Transplantation 47:318-322 (1989)), and are also potential therapeutic agents in the treatment of certain cancers and autoimmune diseases in which the IL-2 receptor plays a role.

IL-2 encoding DNA sequences are reported in a number of publications, and in addition, a modified IL-2-encoding gene, in which a cysteine codon is changed to enhance stability, is described in U.S. Pat. No. 4,518,584, hereby incorporated by reference. U.S.S.N. 834,900, filed Feb. 28, 1986, hereby incorporated by reference, describes a synthetic IL-2-encoding DNA

sequence that differs from the natural IL-2 encoding DNA in that it contains more prokaryotic preferred translation codons than the naturally occurring sequence.

Amino acid deletions or substitutions have been made in the IL-2 amino acid sequence (European Pat. Appln. Nos. 86114468.1 and 87101839.6, U.S. Pat. No. 4,604,377). Although the DNA and amino acid sequences of IL-2 and its crystal structure are known (Brandhuber et al., 1987, Science 238, 1707), there is little data available that allows accurate prediction of the regions of IL-2 that are responsible for biological activity or are sensitive to proteolytic breakdown; e.g., a single substitution of the cysteine residue at position 125 of the IL-2 amino acid sequence with a serine results in increased stability of the molecule (U.S. Patent No. 4,604,377); a substitution of the tryptophan residue at position 121 inactivates the molecule; deletion of amino acid residues 100-104 decreases the biological activity by two oders of magnitude; and deletion of amino acid residues 124-126 renders the molecule inactive (Collins et al., 1988, Proc. Nat. Aca. Sci. 85: 7709; Cohen et al., 1986, Science 234:349).

Summary of the Invention

The present invention provides IL-2 mutant polypeptides that bear a deletion of one to five amino acids, yet retain the ability to bind to IL-2 receptor-bearing cells. It is known that lysine 76 is a proteolytic site in the IL-2 molecule (Cohen et al., 1986, Science 234:349). These mutants either delete this proteolytic site completely, or alter the structure of that area in an effort to reduce proteolysis. The IL-2 mutants can be used as immunostimulants or, when coupled to a toxin to form a hybrid IL2-toxin molecule,

can be used to treat immune and other disorders characterized by the presence of the IL-2 receptor.

The invention thus generally features eight new mutant IL-2 polypeptides capable of binding to the IL-2 receptor; the IL-2 polypeptides have deletions of one or more amino acid residues, as follows: 74; 74-78; 75-77; 76-78; 76-79; 75, 78; and 79 (according to the numbering convention of the Figure, taken from Williams et al., Nucleic Acids Res., vol. 16, no. 22 (1988).

In some preferred embodiments, the mutant IL-2 polypeptide may be part of a fusion protein consisting of a toxin portion (e.g., derived from diphtheria toxin) covalently linked, preferably through a peptide bond at its carboxy terminal end, to the mutant IL-2 polypeptide. The diphtheria toxin portion is large enough to exhibit cytotoxic activity and small enough to fail to exhibit generalized eukaryotic cell binding.

Preferably, the DNA sequence encoding the IL-2 polypeptide contains nucleotide substitutions designed to maximize gene expression in the cells used for expression; i.e., where prokaryotic cells such as E. coli are used, preferred prokaryotic codons are substituted for some of the natural codons (this has been done in the sequence shown in the Figure).

The hybrid molecules of the invention are useful for treating diseases in which the IL-2 receptor plays a role, e.g., IL-2 receptor positive malignancies, allergic reactions, and systemic lupus erythmatosis (SLE), or to prevent an immune response by IL-2 receptor bearing T cells that occurs in graft rejection. This targeted toxin functions by the following mechanism: the IL-2/toxin, by virtue of the IL-2 domain, binds to high affinity IL-2 receptor-bearing cells. The IL-2-toxin is internalized into endocytic vesicles by IL-2 receptor-mediated endocytosis. Acidification of the

endosome causes a conformational change in the toxin, allowing its membrane-associating domains to interact with the endocytic vesicle's membrane and facilitate translocation of the enzymatically active fragment A into the cytosol. Once delivered to the cytosol, fragment A catalyzes the ADP-ribosylation of elongation factor 2, resulting in inhibition of protein synthesis and subsequent death of the IL-2-receptor bearing cell.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawing is first described.

Drawing

The Figure is a DNA sequence, encoding IL-2, in which preferred prokaryotic translation codons are employed; the numbers correspond to the numbering referred to in this specification.

Construction of the Genes Encoding IL-2 Deletion Mutants/Toxin

Amino acids 74 through 79 are contained within the Xbal/Notl fragment of the synthetic IL-2 gene (see Figure). For each of the eight deletion mutants, an Xbal/Notl fragment with a deletion of DNA encoding between one and five amino acids is synthesized using an automated DNA synthesizer according to conventional techniques. The DNA sequences of the oligonucleotides are shown in Table I.

Each Xbal/Notl fragment is synthesized as two complementary strands with a 1/2 Xbal site at the 5' end and a 1/2 Notl site at the 3' end. The synthetic DNA's are gel purified on a denaturing polyacrylamide-urea gel and complementary strands are annealed according to conventional methods. The annealed DNA's are ligated

into the expression plasmid, pDW15 (Williams et al., 1987, Prot. Engineering 1:493), which contains the synthetic IL-2 gene shown in the Figure. Ligation reactions are transformed into a suitable E. coli host according to conventional techniques.

Transformants are screened by restriction digest analysis of minilysate DNA using the restriction enzyme DdeI. The DdeI restriction digest profile of the IL-2 mutants differs from that of non-deleted IL-2 due to elimination of a DdeI site within the XbaI/NotI fragment of the deletion mutants. The DNA sequence of the IL-2 deletion mutants are confirmed by the dideoxy method of Sanger et al. (1977, Proc. Nat. Acad. Sci., 74:5463).

The genes encoding the IL-2/diphtheria toxin fusion proteins are constructed by standard recombinant DNA techniques, as follows. The IL-2 portion of the fusion gene is contained within the SphI/HindIII fragment of the IL-2 deletion mutant derived from pDW15. This DNA fragment is ligated to SphI/HindIII digested plasmid pABM6508 (Bishai et al., 1987, J. Bacteriol, 169:5140), which contains the diphtheria toxin-related portion of the fusion up to and including the amino acid residue Ala 486. The DNA is transformed into a suitable E. coli host and plated onto Luria broth plates plus an appropriate antibiotic for selection, according to conventional techniques. Transformants are screened by DdeI restriction digest analysis of minilysate DNA and by Western blot analy is, as follows.

Western Blot Analysis

Total bacterial cell lysates are analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970, Nature 227:680) for the production of IL-2/toxin protein. Proteins are electroblotted onto nylon

membrane and immunoblot analysis is performed according to conventional techniques. Confirmation of the expected construct is made by positive cross-reactivity to both anti-diphtheria toxin (Connaught Laboratories, Toronto, Ontario, Canada) and to a monoclonal anti-IL-2 antibody, as well as by comparison of the size of the expressed protein to known IL-2/toxin standard. Final confirmation of the construct is made by DNA sequence analysis of the IL-2//toxin gene.

Cytotoxicity assay

Referring to Table II, C91/Pl cells (a high-affinity IL2 receptor-bearing cell line) were seeded in 96-well V-bottom plates (Nunc, Roskilde, Denmark) at a concentration of 10^5 per well in 100 µl complete medium. Il-2-toxin was added at varying concentrations (10^{-12}M to 10^{-6}M) in complete medium. Cells cultured with medium alone were included as the control. Following 18 hours incubation at 37°C in a 5% Co_2 atmosphere, the plates were centrifuged for 5 minutes at 170 x g, the medium was removed and replaced with 100 µl leucine-free medium (DMEM Selectamine, Gibco) containing 2.5 µCi/ml [^{14}C]-leucine (New England Nuclear, Boston, MA). Cells were then incubated at 37° for 90 minutes and collected on glass fiber filters using a cell harvester (Skatron, Sterling, VA). Filters were washed, dried, and counted according to standard methods. All determinations were performed in pentuplicate. IC_{50} refers to the concentration of IL2 required to inhibit protein synthesis to 50% of the untreated control.

IL-2 coding sequence →	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83
(5'→ 3')	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCT	CAG	TCT	AAA	AAC	TTC	CAC	CTG	CGG	CCG	CG
amino acid →		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Gln	Ser	Lys	Asn	Phe	His	Leu	Arg	Pro	
psI 133(Δ74)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	TCT	AAA	AAC	TTC	CAC	CTG	CGG	CCG	CG	
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Ser	Lys	Asn	Phe	His	Leu	Arg	Pro		
psI 134(Δ75)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	CAG	AAA	AAC	TTC	CAC	CTG	CGG	CCG	CG	
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Gln	Lys	Asn	Phe	His	Leu	Arg	Pro		
psI 136(Δ78)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	CAG	TCT	AAA	AAC	CAC	CTG	CGG	CCG	CG	
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Gln	Ser	Lys	Asn	His	Leu	Arg	Pro		
psI 137(Δ79)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	CAG	TCT	AAA	AAC	TTC	CTG	CGG	CCG	CG	
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Gln	Ser	Lys	Asn	Phe	Leu	Arg	Pro		
psI 143(Δ75-77)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	CAG	TTC	CTG	CGG	CCG	CG				
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Gln	Phe	Leu	Arg	Pro					
psI 141(Δ74-78)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	CAC	CTG	CGG	CCG	CG					
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	His	Leu	Arg	Pro						
psI 150(Δ76-79)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	CAG	TCT	CAC	CTG	CGG	CCG	CG			
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Gln	Ser	His	Leu	Arg	Pro				
psI 145(Δ76-78)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	CAG	TCT	CTG	CGG	CCG	CG				
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Gln	Ser	Leu	Arg	Pro					

TABLE I

Table II

Plasmid	amino acid(s) deleted	C91/PL IC50
psI133	Φ74	$6x10^{-1}M$
psI134	Φ75	$1x10^{-10}M$
PsI136	Φ78	$5x10^{-11}M$
psI137	Φ79	$2x10^{-10}M$
psI143	Φ75-77	$2x10^{-10}M$
psI141	Φ74-78	$1x10^{-10}M$
psI145	Φ76-78	$2x10^{-10}M$
psI150	Φ76-79	$7x10^{11}M$
(psI129 control	no deletion	typically $5x10^{-11}M$)

Other Embodiments

Other embodiments are within the following claims. For example, the deletion mutant IL-2 molecules can be used alone, in addition to their use in toxic hybrids, the deletions can advantagously provide resistance to proteolysis in both contexts. In addition, toxins other than diphtheria toxin can be coupled to the mutants, e.g., the enzymatically active portion of Pseudomonas exotoxin can be used.

Claims

1. A mutant IL-2 molecule in which only amino acid residue 74 has been deleted.

2. A mutant IL-2 molecule in which only amino acid residues 74-78 have been deleted.

3. A mutant IL-2 molecule in which only amino acid residues 76-78 have been deleted.

4. A mutant IL-2 molecule in which only amino acid residues 76-79 have been deleted.

5. A mutant IL-2 molecule in which only amino acid residue 75 has been deleted.

6. A mutant IL-2 molecule in which only amino acid residue 78 has been deleted.

7. A mutant IL-2 molecule in which only amino acid residues 75-77 have been deleted.

8. A mutant IL-2 molecule in which only amino acid residue 79 has been deleted.

9. A DNA sequence encoding the mutant IL-2 molecule of any of claims 1-8.

10. The DNA sequence of claim 9, contained in an expression vector.

11. A cell containing the expression vector of claim 10.

12. The DNA sequence of claim 9 wherein said DNA sequence is a synthetic sequence containing more prokaryotic preferred translation codons than naturally occurring IL-2 encoding DNA.

13. A method of producing mutant IL-2 comprising culturing the cell of claim 12 and recovering mutant IL-2 therefrom.

14. The mutant IL-2 molecule of any of claims 1-8, covalently linked to a portion of a toxin molecule which is large enough to exhibit cytotoxic activity and small enough to fail to exhibit generalized eukaryotic cell binding.

15. The molecule of claim 14 wherein said toxin molecule is diptheria toxin, and said portion of diptheria toxin is linked to said mutant IL-2 molecule by a peptide bond.

```
                                1                                  10
      IL-2 amino acid:  Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu Gln
        cDNA sequence:  gca cct act tca agt tct aca aag aaa aca cag cta caa
   codon modifications:                 t   c       c           c     g   g
synthetic DNA sequence: GCA CCT ACT TCT AGC TCT ACC AAG AAA ACC CAG CTG CAG

                           20                                  30
Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile Asn Asn Tyr Lys Asn
ctg gag cat tta ctg ctg gat tta cag alg att ttg aat gga att aat aat tac aag aat
          c c g               g           c c   c   t   c   c               c
CTC GAG CAC CTG CTG CTG GAT TTG CAG ATG ATC CTG AAC GGT ATC AAC AAT TAC AAG AAC
 XhoI

                           40                                  50
Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala Thr Glu Leu
ccc aaa ctc acc agg atg ctc aca ttt aag ttt tac atg ccc aag aag gcc aca gaa ctg
  g       g     c t       g   c   c       c         g               c
CCG AAA CTG ACG CGT ATG CTG ACC TTC AAG TTC TAC ATG CCG AAG AAG GCC ACC GAA CTG
            MluI

                           60                                  70
Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn Leu Ala
aaa cat ctt cag tgt cta gaa gaa gaa ctc aaa cct ctg gag gaa gtg cta aat tta gct
      c   g             g               g       g               t   g   c c g
AAA CAC CTG GAG TGT CTA GAA GAA GAA CTG AAA CCG CTG GAG GAA GTT CTG AAC CTG GCT
                    XbaI
```

FIG. 1-1

```
                                  80                                  90
Gln Ser Lys Asn Phe Hls Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val Ile Val
caa agc aaa aac ttt cac tta aga ccc agg gac tta atc agc aat atc aac gta ata gtt
  g tct           c     c g  ct   g c t     c g     tct   c
CAG TCT AAA AAC TTC CAC CTG CGG CCG CGT GAC CTG ATC TCT AAC ATC AAC GTA ATC GTT
                           NoII

                                 100                                 110
Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala Thr
ctg gaa cta aag gga tct gaa aca aca ttc atg tgt gaa tat gct gat gag aca gca acc
         g       t          c   c                   c                 c
CTG GAA CTG AAG GGC TCT GAA ACC ACC TTC ATG TGT GAA TAC GCT GAT GAG ACC GCA ACC
               BanII

                                 120                                 130
Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe Cys Gln Ser Ile Ile Ser Thr Leu Thr STOP
att gta gaa ttt ctg aac aga tgg att acc ttt tgt caa agc atc atc tca aca cta act tga
  c           c          c t       c       c       g tct           t   c   g   c
ATC GTA GAA TTC CTG AAC CGT TGG ATC ACC TTC TGT CAG TCT ATC ATC TCT ACC CTG ACC TGA
       EcoRI
```

FIG. 1-2

INTERNATIONAL SEARCH REPO

International Application No PCT/US90/04258

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 13/00; C12P 21/02

U.S.CL: 530/351; 435/69.5, 69.52

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	530/351, 435/69.5, 69.52

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [6]

Computer data base search on CAS and dialog for:
IL-2 and delet? and mutat? and amino acids no. 74-79

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	Science, vol. 234, issued 17 October 1986, Cohen et al "Structure-Activity Studies of Interleukin-2," pages 349-51, see page 351.	2-4, 7 1-8
X y	PCT, A, WO/85/00817 (Souza et al), 28 February 1985, see claims.	1-8 **1-8**
X	Science, vol. 238, issued 18 December 1987, Brandhuber et al, "Three Dimensional Structure of Interleukin-2," pages 1707-09, see entire document.	1-8
X	The Journal of Biological Chemistry, vol. 262, No. 12, issued 25 April 1987, Ju et al, "Structure-Function Analysis of Human Interleukin-2", pages 5723-31, see entire document.	1-8

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
6 November 1990	10 JAN 1991
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA / US	Garnette D. Draper, Prim. Exm.

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
X	Gene, vol. 34, issued 1985, Wells et al "Cassette Mutagenesis: An efficient Method for Generation of Multiple Mutations at Defined Sites," pages 315-23, see entire document.	1-8
X	Nucleic Acids Research, vol. 10, No. 20, issued 1982, Zoller et al, "Oligonucleotide-directed Mutagenesis Using M13-derived Vectors: an efficient and General Procedure for the Production of Point Mutations in any Fragment of DNA," pages 6487-6500, see entire document.	1-8

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . ..., because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows: Group I, claims 1-8 to IL-2 muteins, classified 530/351; Group II, claims 9-13, to DNA, vectors, cells and method of making IL-2 mutein, classified 435/69.52 and 172.3; Group III, claims 14-15 to IL-2-toxin conjugates, classified 530/402.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-8 TELEPHONE PRACTICE

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.